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TITLE: Breast Tissue Stromal Cells Preferentially Promote Generation of M2 Macrophages: A Novel Mechanism for Tumor Supportive Properties of Breast Microenvironment

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  Our goals were: (1) to investigate the effect of tissue-specific stromal cells, i.e. mesenchymal stromal/stem cells (MSCs) and macrophages (MQs), on growth of breast tumor cells, and (2) to test the hypothesis that MSCs of non-breast adipose tissues, in contrast to MSCs of breast tissues, precludes such tissues from becoming a site for breast cancer metastasis. We generated MSCs from normal breast and abdominal adipose tissues with phenotypic characteristic similar to bone marrow (BM) MSCs. Only inflammatory cytokine IL1b was expressed at a higher level in abdominal MSCs compared to breast MSCs. MSCs alone, monocyte derived MQs alone, or combined together increased proliferation of MCF7 breast cancer cell line. However, only addition of MQs to BM-MSCs caused a higher level of proliferation of MCF7 cells compared to MSCs alone, suggesting the potential role of BM-MSCs in breast cancer metastasis to bone. MQs co-cultured with breast or abdominal adipose MSCs expressed a higher level of VEGF A, VEGF C, SERPINE1 and FGF2 compared to MQs alone. However, the differences between two MSCs were not statistically significant, possibly because MSCs important in breast cancer growth might not be originating from breast adipose tissue but from ductal/periductal stromal components. Another explanation might be that we derived MSCs from normal, and not cancerous, breast tissues. We propose investigating the biology of subcutaneous adipose tissue, the largest human tissue and the least receptive to breast cancer metastasis, as a novel approach to find more effective therapeutic options for breast cancer.					
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**Introduction:** Breast cancer tissue is rich in stromal components including mesenchymal stromal/stem cells (MSCs) (1, 2) and macrophages (3, 4); both of these cells are assumed to play a significant role in progression of breast cancer through their interactions with tumor cells. Although there is much research on breast cancer tumor microenvironment with the goal of investigating the role of tumor associated macrophages and stromal cells in growth of breast cancer cells, there is not much known about the interactions between these two important cellular constituents of breast cancer microenvironment. Research in our laboratory is focused on interactions between MSCs and macrophages in normal and malignant tissues. In contrast to all studies focused on the role of breast tissue microenvironment in growth of primary breast cancer cells and investigation of why some tissues/organs (such as bone marrow, lungs, brain and liver) (5)) are prone to be sites of breast cancer metastasis, we are investigating why subcutaneous adipose tissue-one of the largest tissues in human body (6) and a tissue very rich in both MSCs and macrophages-is an extremely uncommon site for metastatic breast cancer (7, 8). However, we are also cognizant of the fact that obesity is considered to be a risk factor for breast cancer development (9). We hypothesized that MSCs resident in breast tissue preferentially convert breast tissue macrophages into an immunophenotype favorably supporting growth of breast cancer cells, and conversely that MSCs and macrophages in adipose tissues provide an inhospitable microenvironment for growth of tumor cells.

## **Body**

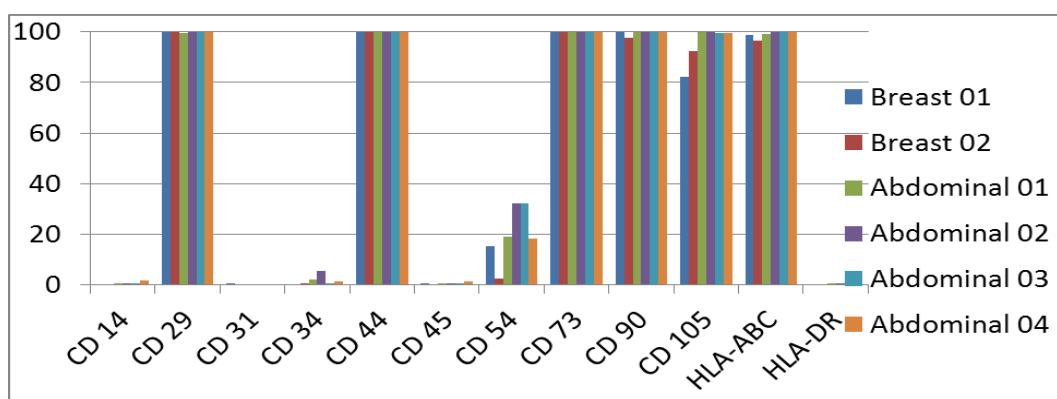
Our DoD proposal was designed to investigate two specific aims: 1) To determine the phenotype/genotype of MSCs and macrophages isolated from breast and abdominal fat, and 2) To determine the effect of MSCs isolated from breast and abdominal fat on the phenotype of macrophages. Our goal was to test the hypothesis that MSCs of breast adipose tissue origin, through changing the phenotype of macrophages, provide an immune environment suitable for growth of breast cancer cells, but MSCs present in non-breast adipose tissues precludes such tissues to become a site of metastasis for breast cancer, through converting macrophages into inflammatory (anti-tumor) macrophages. While we were successful to generate MSC lines from breast and abdominal adipose tissue we were not successful in isolating macrophages from these tissues. However, in our laboratory we simulated the microenvironment of breast and non-breast adipose tissues by co-culturing MSCs (derived from these tissues) with blood-derived monocytes and investigated both the phenotype of generated macrophages and their effects on growth of breast cancer cell lines.

## **Results**

### **I) Generation and characterization of abdominal and breast adipose tissue-derived MSCs**

Breast adipose tissues were collected from normal healthy females undergoing mammary reduction surgeries, and abdominal adipose tissues were collected from normal healthy females undergoing abdominoplasty surgery. All collections were based on IRB approved protocols. MSCs were generated based on standard protocols, passaged until passage 4, and then were tested for phenotypic characteristics of MSCs.

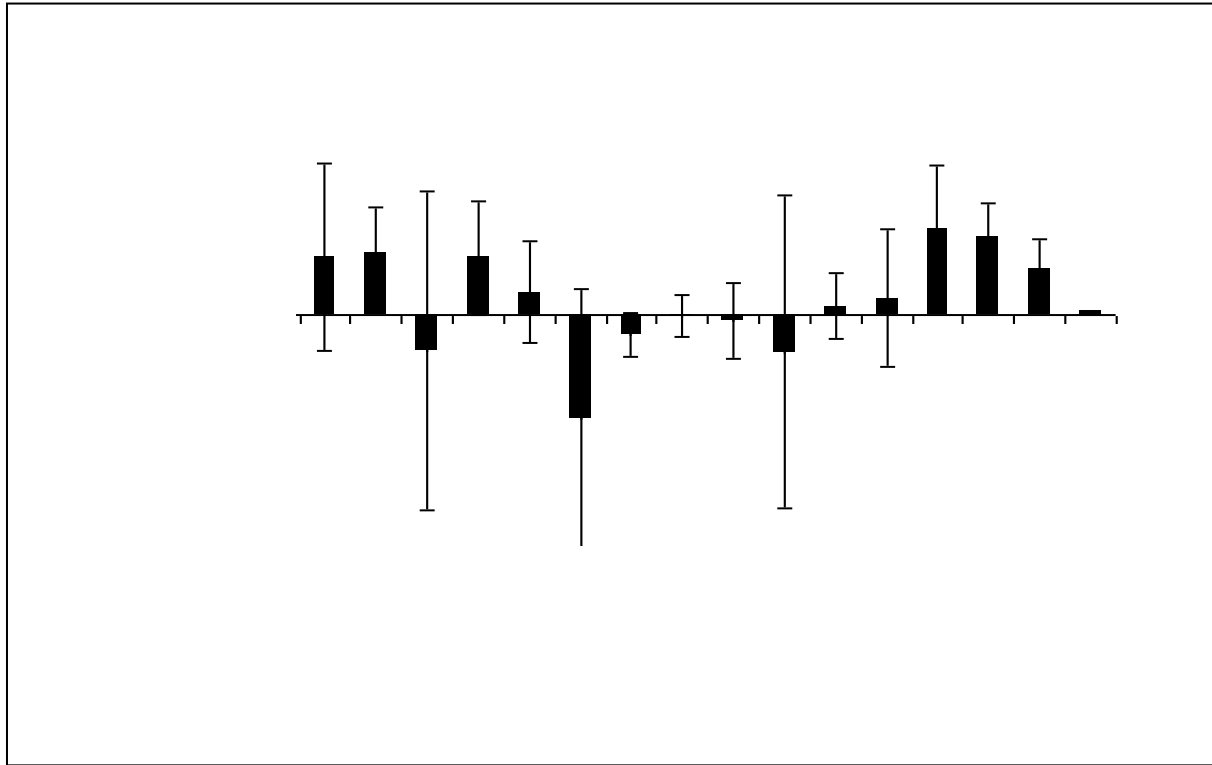
Differentiation assays for adipogenic, osteogenic and chondrogenic potential of MSCs were done using AdipoDiff Media, OsteoDiff Media and ChondroDiff Media (Miltenyi Biotec) according to manufacturer's protocols. We verified the tri-lineage potential of abdominal adipose and breast adipose derived MSCs (data not shown). Cell surface marker expression of these MSCs were analyzed using Accuri C6 flow cytometer and CFlow plus software (Accuri Cytometer). **Figure-1** shows that these MSCs (four abdominal adipose-derived MSC lines and two breast adipose-derived MSC lines) exhibit a cell surface marker expression pattern similar to what has been reported for bone marrow derived MSCs. Abdominal and breast adipose tissue-derived MSC lines express CD29, CD73, CD90, CD105, CD44 and HLA-ABC, while being negative for CD14, CD31, CD34, CD45, CD54 and HLA-DR markers. These cell surface marker characteristics define these cells as MSCs according to accepted criteria for definition of MSCs (10).



**Figure-I** Cell surface marker expression of MSCs derived from abdominal versus breast adipose tissues

## II) Comparison of gene expression of breast and abdominal adipose tissue derived MSCs.

Real time qPCR analysis was performed to compare gene expression levels of genes potentially important in breast cancer growth between 2 breast and 4 abdominal adipose-derived MSCs. To our surprise, among the genes tested, IL1b was the only gene expressed at a significantly higher level in abdominal adipose MSCs compared to breast adipose MSCs (see Figure II). IL1b is an inflammatory cytokine, and we speculate that its higher secretion by adipose tissue derived MSCs could provide an inhospitable environment for metastasis in adipose tissue. However, IL1b has also been proposed as a tumor promoting cytokine. Interestingly, in the scientific literature, macrophages and not stromal cells have been proposed as the main source of IL1b.



**Figure-II** Comparison of gene expression between MSCs derived from abdominal or breast adipose tissues

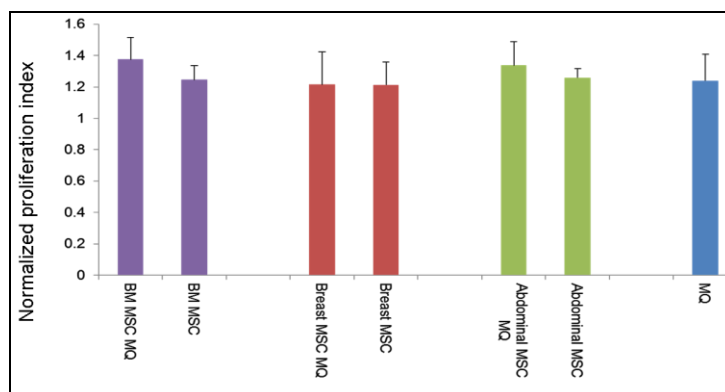
### III) MCF7 breast cancer cell line proliferation assay

In this set of experiments, we investigated the effect of different lines of MSCs-alone or in combination with macrophages-on growth of MCF7 breast cancer cell line, a widely used cell line for study of breast cancer biology (11, 12). MSCs were plated into 6 well plates at a concentration of 100,000 cells per well. In the case of MSC-macrophage co-culture, MSCs were added to macrophage plates prepared by culturing CD14 positive cells for 5 to 7 days in IMDM media supplemented with 10% human serum type AB. MCF7 cells were then stained with CFSE (13) and then added to plates at 100,000 cells per well. After a 3 to 4 day co-culture, all the cells were harvested, and proliferation of MCF7 cells was analyzed by ModFit software (version 3.1).

#### III-A) Effect of co-culturing MSCs and/or macrophages on proliferation of MCF7 cells

MSCs alone, macrophages alone, or combined together increased proliferation of MCF7 cells compared to no extra added cells. However, only addition of macrophages to bone marrow derived MSCs caused a significantly higher level of proliferation of MCF7 cells compared to MSCs alone. While these experiments could not show a preferential effect of breast adipose tissue derived MSCs on growth of MCF7 cells it is consistent with previous observations that bone marrow derived MSCs play a major role in breast cancer metastasis (14-16). The lack of difference between breast and abdominal adipose MSCs could be due to the fact that with our isolation methodology

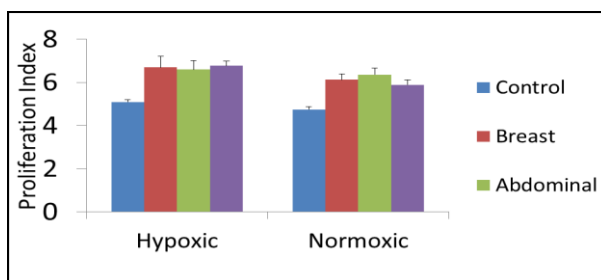
we were not able to isolate the actual MSCs (i.e. ductal and peri-ductal) responsible for promoting growth of breast cancer cells (17).



**Figure-III-A** Effect of co-culturing different types of MSCs alone or in combination with macrophages on growth of MCF7 cell line using proliferation index as measured by CFSE

### III-B) Effect of hypoxia on proliferation of MCF7 breast cancer cell line with MSCs

Presence of MSCs in normoxic conditions (21% O<sub>2</sub>), regardless of their origin, increased proliferation of MCF7 cells (see Figure III-B). MCF7 cells proliferated even more in hypoxic condition (5% O<sub>2</sub>) compared to normoxic condition. However, breast and abdominal adipose tissue derived MSCs did not show a difference in their capability to support proliferation of MCF7 cells. These studies verify the role of hypoxia in tumor progression (18).

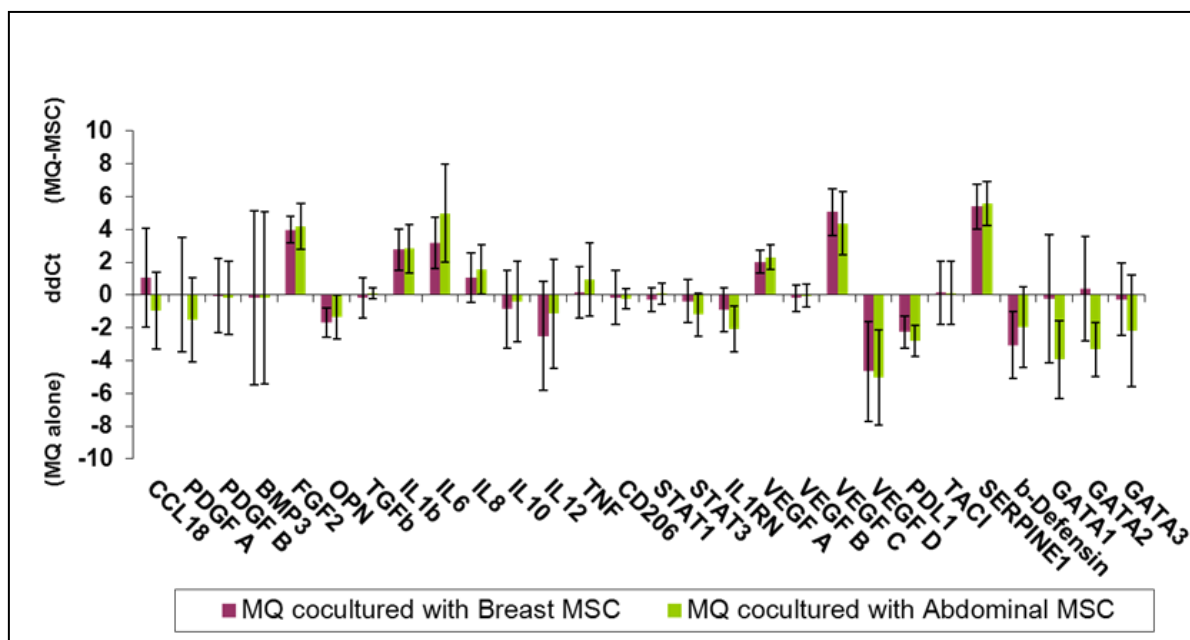


**Figure-III-B** Effect of hypoxic and normoxic condition of growth of MCF7 cells co-cultured with different MSCs

### IV-Changes in gene expression of macrophages co-cultured with breast and abdominal adipose MSCs versus control macrophages

We next examined the expression of genes, potentially important in breast cancer progression, using qPCR by macrophages after their co-culture with breast or abdominal adipose tissue derived MSCs, respectively, compared to macrophages cultured alone. Although macrophages co-cultured with MSCs expressed a higher level of VEGF A, VEGF C, SERPINE1 and FGF2 compared to macrophages alone, the levels of expression of these genes were not statistically significant between breast and abdominal adipose tissue derived MSCs (see Figure IV). We were surprised that both

abdominal and breast adipose tissue derived MSCs expressed similar levels of genes important in breast cancer growth; however, as explained above this might be due to the fact that MSCs important in breast cancer growth might not be originating from adipose tissue present in breast but from MSCs of ductal and peri-ductal origin. Another explanation might be that we derived MSCs from normal, and not cancerous, breast tissues (19).



**Figure-IV** Changes in gene expression pattern of macrophages after co-culturing with breast or abdominal adipose tissue derived MSCs compared to macrophages cultured alone

### Key Research Accomplishments:

- 1) Generation and phenotypic and genotypic characterization of MSC lines derived from human breast and abdominal adipose tissues
- 2) Determination of effect of co-culturing MSCs with or without macrophages on growth of MCF7 breast cancer cell line
- 3) Determination of effect of co-culturing different types of MSCs on macrophages' expression of genes important in breast cancer growth

### Reportable Outcomes

Our work as described in this final report was presented as a poster at Department of Defense, Breast Cancer Research Program, Era of Hope Conference, August 2011; Orlando, Florida. We are preparing a manuscript to describe our findings for publication in a peer review cancer journal.

### Conclusion

The goal of our study was to investigate the effect of tissue specific stromal cells on growth of breast tumor cells. We characterized mesenchymal stromal/stem cells

(MSCs) derived from breast and abdominal adipose tissues, and compared their effects on proliferation of MCF7 breast cancer cell line alone or with human monocyte derived macrophages. To our surprise, our results showed that breast and abdominal adipose-derived MSCs are equivalent in terms of supporting MCF7 breast cancer cell line proliferation, either alone or in combination with macrophages. Also both MSC types similarly induce expression of genes important in breast cancer growth in macrophages such as VEGF A, VEGF C, SERPINE1 and FGF2. This could be due to the following: (a) the fact that our breast adipose-derived MSCs might not be truly representative of MSCs that are present in breast tissues in the vicinity of tumor cells and responsible for supporting their growth, or (b) the fact that we derived MSCs from normal, and not cancerous, breast tissues. However, in our experiments addition of macrophages to bone marrow MSCs showed a statistically significant synergistic effect on promoting proliferation of MCF7 cell line, thereby providing a potential explanation as to why bone is such a common site of metastasis for advanced breast cancer. **Significance:** There is much interest and resources dedicated to investigate why breast tissue is supportive of growth of primary breast tumor and why metastasis prone tissues (such as bone marrow, lungs, brain and liver) are receptive to breast cancer metastatic cells. We propose that investigating the biology of subcutaneous adipose tissue-the largest tissue in human body but at the same time the least receptive tissue to breast cancer metastasis-could provide a novel approach to finding effective therapeutic options for breast cancer (20-22).

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## Appendices:

None